HIV-Specific, *Ex Vivo* Expanded T Cell Therapy: Feasibility, Safety, and Efficacy in ART-Suppressed HIV-Infected Individuals

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Adoptive T cell therapy has had dramatic successes in the treatment of virus-related malignancies and infections following hematopoietic stem cell transplantation. We adapted this method to produce *ex vivo* expanded HIV-specific T cells (HXTCs), with the long-term goal of using HXTCs as part of strategies to clear persistent HIV infection. In this phase 1 proof-of-concept study (NCT02208167), we administered HXTCs to antiretroviral therapy (ART)-suppressed, HIV-infected participants. Participants received two infusions of 2×10^7 cells/m^2 HXTCs at a 2-week interval. Leukapheresis was performed at baseline and 12 weeks post-infusion to measure the frequency of resting cell infection by the quantitative viral outgrowth assay (QVOA). Overall, participants tolerated HXTCs, with only grade 1 adverse events (AEs) related to HXTCs. Two of six participants exhibited a detectable increase in CD8 T cell-mediated antiviral activity following the two infusions in some, but not all, assays. As expected, however, in the absence of a latency reversing agent, no meaningful decline in the frequency of resting CD4 T cell infection was detected. HXTC therapy in ART-suppressed, HIV-infected individuals appears safe and well tolerated, without any clinical signs of immune activation, likely due to the low residual HIV antigen burden present during ART.

INTRODUCTION

HIV persists within a latent reservoir in transcriptionally quiescent cells, unaffected by antiretroviral therapy (ART) and of limited visibility to the immune system.[1–3] The long half-life of these cells suggests that more than 70 years of ART would be required to eradicate this latent HIV reservoir.[4,5] Approaches using pharmacological HIV latency reversal agents (LRAs) to induce latent virus to express viral protein may make this reservoir vulnerable to CD8 T cells and other effector mechanisms.[6–11]

HIV-reactive CD8 T cells play a crucial role in the control of HIV viremia.[12–14] However, the existing HIV-specific immune response in ART-suppressed individuals is insufficient to clear persistent infection, even in the presence of LRAs that induce HIV expression.[15–17] Obstacles to immune-mediated clearance of reactivated latent HIV include the low frequency of HIV-specific T cells in ART-suppressed individuals, impaired CD4 T cell help, pre-existing immune escape variants archived in the latent reservoir, and dysfunctional CD8 T cell function dominated by responses that lack specificity for these escape variants.[11,18–21]

Adoptive T cell therapy using autologous T cells that are expanded *ex vivo* in the presence of viral peptides and cytokines offers a safe avenue for reeducation and expansion of T cell responses through precise manipulation of cell number, phenotype, and function that would not otherwise be safe or feasible *in vivo*.[22] Earlier adoptive T cell therapy approaches for HIV had limited efficacy because of multiple factors, including use of single epitope specificity, the development of escape variants in actively viremic participants, and effector cell exhaustion following *ex vivo* expansion.[22–27] Since these earlier attempts, the adoptive T cell therapy field has made significant advances, largely in the oncology field, that may be leveraged to overcome some of the pitfalls encountered with earlier T cell therapy approaches for HIV. Polyclonal, virus-specific T cells expanded *ex vivo* and reinfused into hematopoietic stem cell transplant patients have shown efficacy for the treatment of Epstein-Barr virus (EBV), cytomegalovirus (CMV), adenovirus, BK virus, and human herpesvirus 6 infection[28–34] as well as the treatment of EBV-mediated malignancies.[35–39] T cells

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Table 1. Clinical Characteristics and Baseline Reservoir Size

Study ID	Age (Years)	Sex	Race/Ethnicity	Stage ART Initiated[a]	Nadir CD4	CD4 at Study Entry	Pre-ART Viral Load	Years on ART	IUPM[b]
HXTC-02	50	M	Caucasian	chronic	168	563	323,000	8	0.21
HXTC-03	35	M	Caucasian	acute	427	721	24,905	7	<0.02
HXTC-04	65	M	Caucasian	acute	277	950	14,538,865	9.5	0.90
HXTC-05	53	M	Caucasian	chronic	174	1,047	635,000	2.5	2.49
HXTC-06	61	F	African American	chronic	528	943	18,910	1	0.24
HXTC-07	58	M	Caucasian	chronic	338	816	58,500	6	0.89

All values were taken at the time of screening or baseline leukapheresis.

[a]Acute HIV infection (AHI) was defined as: (1) a negative or indeterminate enzyme immunoassay (EIA) plus a reproducibly detectable HIV by amplification methods, or (2) a positive fourth-generation HIV Ag/Ab combination assay and either a negative or indeterminate HIV rapid test or a negative/indeterminate western blot, or (3) a negative HIV RNA test within 45 days of a positive EIA and ART initiation. All others with confirmed HIV infection on ART were defined as having started HIV therapy during chronic infection.

[b]IUPM is infectious units per million, as measured by the quantitative outgrowth assay (QVOA) following maximal mitogenic stimulation with PHA as previously described.[45]

generated using these sophisticated methods of expansion have been safe and well tolerated, as well as highly effective.[38,40]

We previously demonstrated that it is feasible to adapt this method of expansion of polyclonal virus-specific T cells to the HIV setting and expand polyfunctional, potent HXTCs targeting multiple HIV epitopes under good manufacturing practice (GMP) conditions.[41–43] In this study, we demonstrate the *in vivo* safety and tolerability of HXTC therapy in HIV-infected, ART-suppressed participants.

RESULTS

Clinical Characteristics and Background of Participants

All participants were on a stable regimen of suppressive ART at the time of study entry, with median duration of ART of 6.5 (range 1–9.5) years (Table 1). Participants were mostly male (five of the six participants) and Caucasian (five of six participants), with one female African American participant, and had a median age of 55.5 (range 35–65) years (Table 1). Two of the six participants initiated ART during acute infection.[44] Nadir CD4 ranged from 168 to 528, with a median of 277, although all participants had experienced CD4 recovery following ART initiation. Reflecting the range in baseline clinical and virologic characteristics, the frequency of persistent resting CD4 T cell infection ("reservoir size") in infected units per million (IUPM) as measured by quantitative outgrowth assay (QVOA[45]) ranged from below the limit of detection of the assay (<0.02 per million in HXTC-03) to 2.49 per million, with a median IUPM of 0.56, similar to those previously reported.[4,11]

HLA typing by sequencing was performed for all participants (Table S1). One participant, HXTC-06, was found to have two HLA alleles known to be favorable host factors, HLA-B*81:01 and HLA-A*74:01.[46–50] In contrast, HLA alleles associated with ineffective viral control and rapid disease progression, HLA-B*18, HLA-B*35, and HLA-B*08, were found in HXTC-02, -04, and -05, respectively.[51,52]

HXTC Product Phenotype and Specificity

HXTCs were generated as previously reported, using lymphocytes stimulated by irradiated autologous dendritic cells pulsed with overlapping HIV peptides spanning the entire regions of Gag, Pol, and Nef in the presence of supportive cytokines and then stimulated by pep-mix-pulsed autologous phytohemagglutinin (PHA)-treated lymphoblasts (Figure S1).[41,42] All cultures were performed in the presence of the antiretroviral agents indinavir and raltegravir.

Using this expansion method, HXTCs were readily expanded to numbers sufficient for use in the clinical trial after just two rounds of stimulation in just over 3 weeks (Figure 1A). The total fold expansion starting with bulk lymphocytes ranged from approximately 2.5 logs (HXTC-04) to greater than 3 logs expansion, in the cases of HXTC-02 and HXTC-06. As previously published, expansion is exponential, with a significant augmentation of expansion following the second stimulation.[41,42] HXTCs exhibited specificity for the Gag, Nef, and Pol peptide pools as measured using interferon gamma (IFNγ) ELISpot assays, with negligible nonspecific activity (Figure 1B). The magnitude and breadth of HIV-specific T cell responses within the HXTC product varied among participants. HXTCs from three of the six participants showed T cell responses to all three peptide pools (HXTC-05, -06, and -07), two exhibited T cell responses to two of the three peptide pools (HXTC-02 to Nef and Pol, and HXTC-03 to Gag and Pol), while HXTCs from one participant had a T cell response targeting Nef only (HXTC-04). The median magnitude of T cell responses was 146.0, 237.9, 549.1, and 650.58 IFNγ spot-forming centers (SFCs)/10^5 cells to Gag, Pol, Nef, and combined peptide pools, respectively.

HXTCs were predominantly CD8 T cells (median 72%, range 39%–88%), although natural killer (NK) cells expanded within the HXTCs from some participants, with the largest proportion seen in HXTC-07 (21.8%) and HXTC-04 (36.1%; Figure 1C). As expected, there were no residual dendritic cells or co-stimulatory K562 cells detected at the end of the expansion period (Figure 1C). Despite the two rounds of stimulation, HXTCs displayed detectable but low levels of immune exhaustion markers (Figure 1D).

HXTCs also demonstrated potent cytotoxicity against autologous target cells pulsed with Gag, Pol, and Nef peptide pools in ^{51}Cr release assays (Figure 1E). Cytotoxicity was HIV-specific, with negligible ^{51}Cr

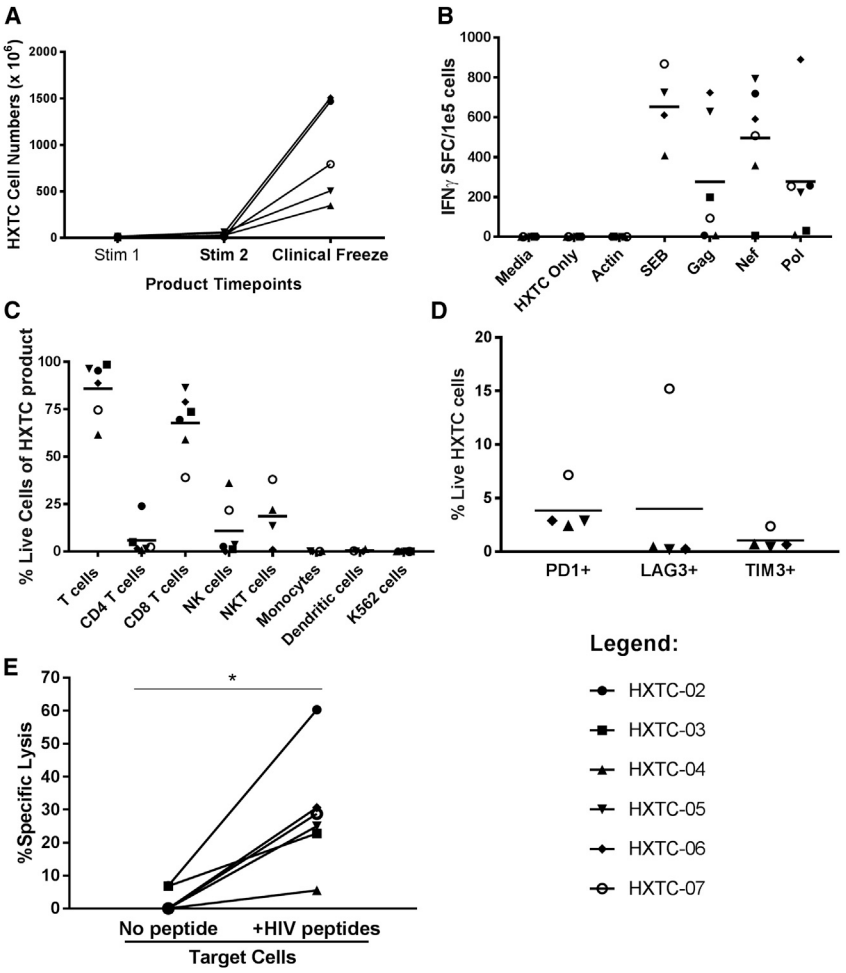

Figure 1. Characterization of HXTC Products

(A) HXTC products expanded to clinically relevant levels after two stimulations (n = 6). (B) All HXTC products produced IFNγ in response to HIV Gag, Neg, Pol (GNP) pepmix stimulation, as measured on ELISpot. (C) Product phenotying by flow to show T cells ($CD45^+$, $CD3^+$), CD4 T cells ($CD3^+CD4^+$), $CD8^+$ T cells ($CD3^+CD8^+$), NK cells ($CD3^-CD56^+CD16^+$), natural killer T (NKT) cells ($CD3^+CD56^+CD16^+$), monocytes ($CD45^+CD14^+$), dendritic cells ($CD3^-CD83^+$, $HLADR^+$), and K562 cells ($CD45^+CD562^-$, $CD16^-$, $CD56^-$, $CD32^+$, $CD83^+$). (D) HXTC products displayed minimal exhaustion on $CD3^+$ T cells. (E) ^{51}Chromium cytotoxicity assay shows specific lysis of HXTC products against autologous PHA blast target cells pulsed with no peptides or HIV peptides (gag, nef, pol peptide pools) at an E:T ratio of 20:1. Each data point represents the mean of three technical replicates. *$p < 0.05$ by Wilcoxon signed rank test.

release seen when HXTCs were co-cultured with autologous target cells that were not pulsed with any peptide (Figure 1E). Maximum HIV-specific cytotoxicity ranged from approximately 8% specific lysis (HXTC-04) to greater than 60% specific lysis (HXTC-02), with a median cytotoxicity of 24%, at an effector:target (E:T) ratio of 20:1 (Figure 1E). Little difference was seen at ratios as low as 5:1, except for HXTC-04, which exhibited a lower maximum cytotoxicity than HXTCs from other participants and did show a dose-dependent cytotoxicity, with 4% specific lysis at the lowest E:T ratio tested (5:1), up to a maximum of 8% specific lysis at higher E:T ratios.

HXTC Expression of Homing Markers to Gut and Lymph Node Tissues

Homing markers, including integrin α4β7, L-selectin (CD62L), and the chemokine receptor CXCR5, were examined to determine whether HXTCs exhibited the potential to home to putative sites of persistent HIV infection, including the B cell follicle and the gut. Memory $CD8^+$ T cells (live, dump $channel^-$, $CD3^+$, $CD4^-$, $CD8^+$, $CD45RO^+$; Figure 2A for gating strategy) from three HXTC participants were evaluated, chosen based on cell availability. Integrin α4β7 was expressed at a range of frequencies on baseline peripheral blood mononuclear cells (PBMCs; mean of 31.25% of memory CD8 T cells, range 12.85%–51.2%), and trended to an increase in frequency in HXTCs (mean 78% of memory CD8 T cells, range 41.4%–97.5%) (Figure 2B). CD62L and CXCR5 were expressed on a minority of peripheral blood memory T cells (mean 21.1%, range 14.0%–31.3% $CD62L^+$ CD8 memory T cells; mean 2.8%, range 1.2%–4.2% $CXCR5^+$ CD8 memory T cells), and expression either remained stable or trended to a decrease on HXTCs compared with PBMCs (Figure 2B).

HXTC Infusion Was Safe and Well Tolerated

All six participants completed the study protocol consisting of two HXTC infusions (2 × $10e7/m^2$/dose) 2 weeks apart, without dose-limiting toxicity. Overall, the cell infusions were safe and well tolerated. Two participants (HXTC-02 and HXTC-07) had transient, self-limiting fevers and myalgia of DAIDs toxicity grade 1 severity 1 day post-infusion (HXTC-02) and 2 months post-infusion (HXTC-07). One participant (HXTC-07) also experienced a transient increase in viremia above the 40 copies/mL limit of detection 1 month following infusion, with a peak detectable viral load of 80 copies/mL that resolved without intervention within 60 days. No other treatment-related adverse events occurred. Other adverse events recorded were not attributable to the HXTC infusion. This included a transient increase in blood pressure with the leukapheresis procedure in four of six participants, an expected and well-described effect of the procedure, all at or below grade 3.

Antiviral Activity as Measured by a VIA and Latency Clearance Assay following HXTC Infusion

We next determined whether the antiviral activity of CD8 T cells isolated from PBMCs of participants was altered following HXTC infusion using a viral inhibition assay (VIA) in which autologous

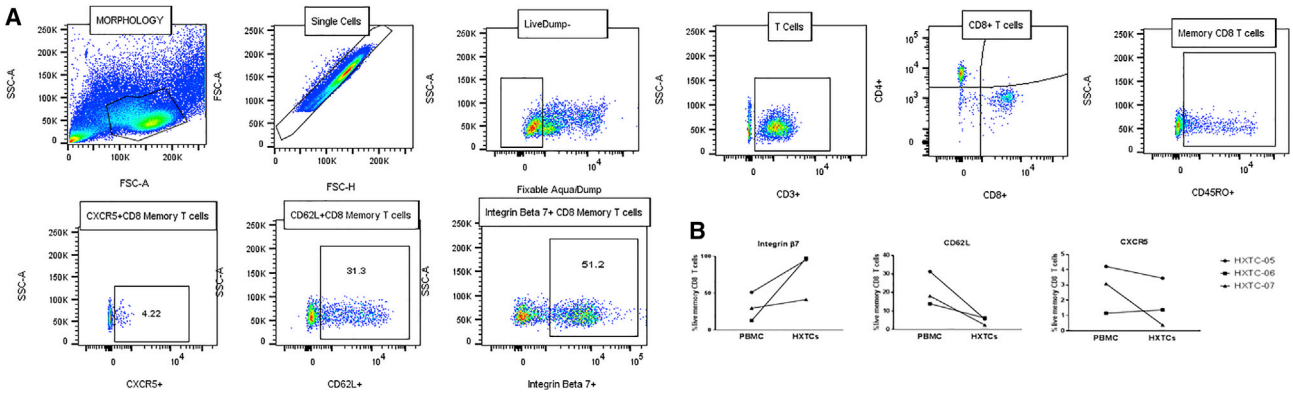

Figure 2. Homing Markers of PBMCs and HXTCs

PBMCs and HXTCs from three participants chosen based on cell availability were thawed, rested overnight, and stained for the described cell surface markers. Gating strategy is shown in (A). The % of live, $CD8^+$ memory T cells for the indicated homing marker is shown for both PBMCs and HXTCs (B). Data points represent the mean of technical duplicates.

activated CD4 T cells are spinoculated with the lab-adapted, CCR5 tropic HIV-1 isolate JR-CSF and co-cultured with CD8 T cells at a low E:T ratio of 1:10. A decrease in the p24 production measured in supernatant 6 days later indicates inhibition of viral spread. CD8 T cell performance in this assay may reflect multiple antiviral functions, including proliferation, cytokine production, and cytotoxicity, and has been associated with parameters of *in vivo* viremic control.[53] Baseline antiviral activity varied among participants and ranged from modest reduction of p24 production, to 50% of that seen in the absence of CD8 T cell addition, to no reduction (Figure 3A).

Two participants (HXTC-02, HXTC-07) exhibited a mild to modest and sustained improvement in antiviral activity, with a reduction in the %p24 production in this assay seen as early as 1 week following the first infusion (Figures 3A–3F). The improved ability to reduce p24 production was maintained through the last time point measured, 13 weeks following the first infusion (Figures 3A and 3F). However, no impact on antiviral activity was seen in the remaining four participants (Figures 3B–3E); in aggregate across all six participants, no significant improvement in antiviral activity was observed following HXTC infusion.

To measure the antiviral activity in an assay with potentially more clinical relevance for participants on ART, during which time HIV antigen exposure is likely rare and at very low levels, we performed latency clearance assays (LCAs).[42,54,55] In this assay, autologous resting CD4 T cells were exposed to vorinostat, a histone deacetylase inhibitor under clinical investigation as a LRA,[6,8,9,43,56,57] and then co-cultured with CD8 T cells for a discrete 24-hr period. CD8 T cells are then depleted so that reductions in virus recovery are attributed to the discrete co-culture period only, and allogeneic activated CD8-depleted PBMCs are added to amplify any residual rare virus in replicate wells plated under limiting dilution conditions. In two of the six participants, the frequency of latent virus infection was too low at baseline to perform this assay with the cells available. The participant with the strongest improved CD8 performance in the VIA (HXTC-02) also showed an improved activity of CD8 T cells in the LCA following HXTC infusion, from modest (50%) reduction in virus recovery with co-culture with pre-infusion CD8 T cells to complete ablation of virus recovery with co-culture with post-infusion CD8 T ($p = 0.01$ by Fisher's exact test). Although a trend toward reduction was seen for the other three participants, it was not statistically significant due to the low number of positive wells in the control condition (three or fewer positive wells in control condition). The total number of wells plated was restricted due to limited cell availability and the large number of cells required for the assay, a known limitation of the assay,[43] preventing definitive conclusions from being drawn. The week 13 post-infusion time point evaluated was chosen based on cell availability from the leukapheresis procedure; earlier post-infusion time points were not assessed.

The Frequency of HIV-Specific T Cells as Measured by IFNγ Release Did Not Change following HXTC Infusion

The impact of HXTC infusion on *ex vivo* HIV-specific T cell responses was measured by IFN-γ ELISpot. HIV reactive T cell responses were detectable in all six participants at baseline. No increase or change in T cell specificity was observed following HXTC infusion.

IFNγ release from T cells in response to clade B consensus peptide pools of Gag, Pol, or Nef was detectable for at least one of the three peptide pools in all six participants at baseline, with a median of 61.5 SFU/10^5 PBMCs to Gag (Figure 4A), a median of 58.8 SFU/10^5 PBMCs to Pol (Figure 4B), and a median of 47.7 SFU/10^5 PBMCs to Nef (Figure 4C). Overall, despite the increase in antiviral activity detected in two of the six participants, no significant change in IFNγ release to Gag, Nef, or Pol peptide pools was observed when measured at 1, 2, 4, 8, and 13 weeks following the first HXTC infusion (results shown for week 13).

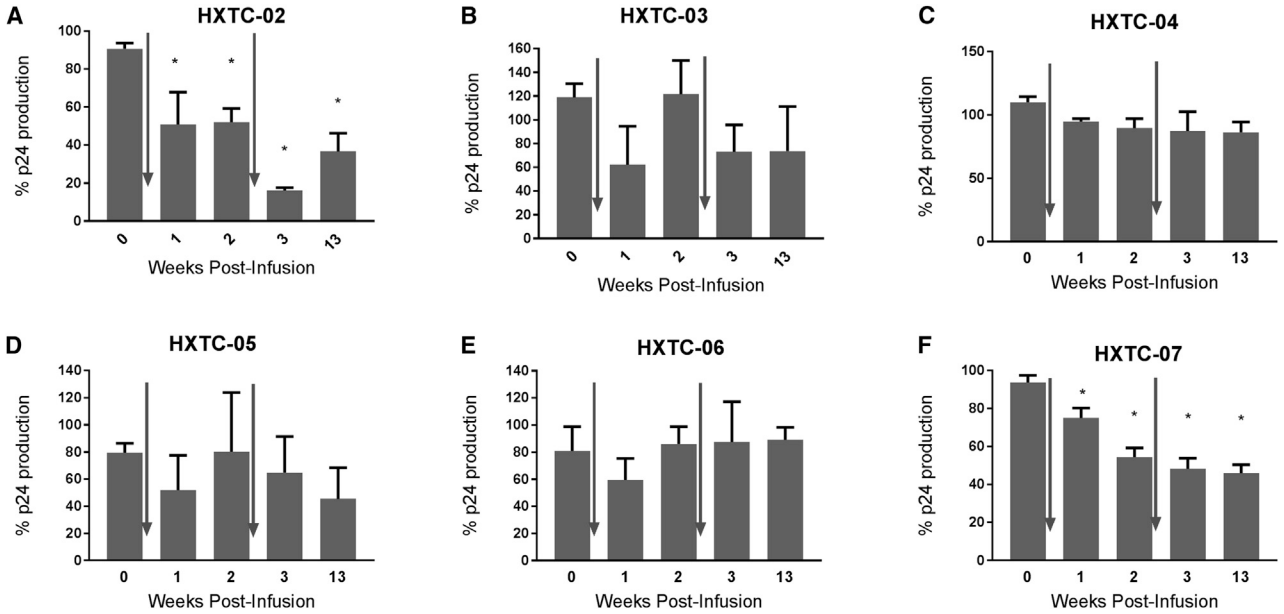

Figure 3. Antiviral Activity following HXTC Infusion

(A–F) Viral inhibition assay against autologous activated CD4 T cells infected with JR-CSF and co-cultured with CD8 T cells at an effector:target ratio of 1:10 obtained from participants at baseline (averaged from 4 and 0 weeks before infusion) and 1, 2, 3, and 13 weeks post-infusion. HXTC-02 (A), HXTC-03 (B), HXTC-04 (C), HXTC-05 (D), HXTC-06 (E), HXTC-07 (F). Infusions occurred at weeks 0 and 2, denoted by arrows. Results are displayed as %p24 production, normalized to the p24 obtained in the absence of any CD8 effector cells. Error bars represent SEM of n = 4 replicates per time point per participant. Arrows denote HXTC infusion. *$p < 0.05$ by one-way ANOVA with Dunnett's post-test correction for multiple comparisons.

Virologic Outcome following HXTC Infusion

We next assayed the frequency of rare, latently infected resting CD4 T cells via the quantitative viral outgrowth assay (QVOA) using maximally activated resting CD4 T cells, as previously described.[4] Overall, no change in the frequency of latently infected resting CD4 T cells was observed following HXTC infusion, indicating no detectable impact on the latent reservoir (Figure 5A; $p = 0.13$ by Wilcoxon matched-pairs signed rank test). To assess the effect of HXTC infusion on persistent, residual low-level viremia, we measured the plasma load of HIV using a single-copy assay (SCA) capable of detecting plasma RNA levels lower than 1 copy/mL.[58] Two baseline SCA values were obtained prior to infusion; then plasma samples were analyzed at weeks 3 and 13 following the first infusion. Five of six participants had detectable low-level viremia at baseline, with a median of 2 copies/mL (range 0.74–5.5), consistent with what has been reported.[59] No significant change in low-level, residual viremia was detected (Figure 5B; $p = 0.58$ by Friedman's test). High variability of SCA results for participants from visit to visit was observed and was consistent with results from longitudinal studies of ART-suppressed individuals.[59] The high visit-to-visit variability would prevent detection of more modest reductions.

DISCUSSION

HXTC infusions were safe and well tolerated in HIV-infected, ART-suppressed individuals, with few mild treatment-related adverse events (grade ≤ 1). The safety and tolerability in this first proof-of-concept study for HXTC therapy is consistent with results of previous clinical trials of adoptively transferred virus-specific T cells, with most studies documenting only grade 1 or 2 adverse events.[40]

Two of six participants demonstrated an increase in CD8 T cell antiviral activity against JR-CSF HIV-infected autologous targets in an *ex vivo* autologous assay (VIA), although the clinical significance of this mild to modest impact is unknown. Interestingly, these were the participants who reported mild flu-like illnesses following infusion. This increase in antiviral activity did not correlate with an increase in the apparent frequency of HIV-specific T cells as measured by IFNγ release following stimulation with Gag, Pol, and Nef peptide pools. This discrepancy may be explained by underlying differences in the assays. IFNγ release following overnight stimulation measures only one aspect of antiviral function over a short time period. In contrast, VIAs span 6 days of co-culture, and thus may reflect multiple antiviral functions including proliferation, cytokine release, and cytotoxicity. HXTC product of the participant with the strongest increase in antiviral activity (HXTC-02) did exhibit by far the strongest HIV-specific cytotoxicity. Importantly, antiviral activity as measured in a VIA has been associated with *in vivo* parameters of viremic control;[53] however, the robustness and utility of such assays in the setting of efforts to clear persistent HIV infection require further study and validation. Further evaluations into the mechanisms underlying this difference in antiviral capacity, including the breadth of HIV-specific responses and functional avidity of the TCRs, will be important to include in analysis if this signal is replicated in larger numbers as additional participants receive HXTC infusions in ongoing studies.

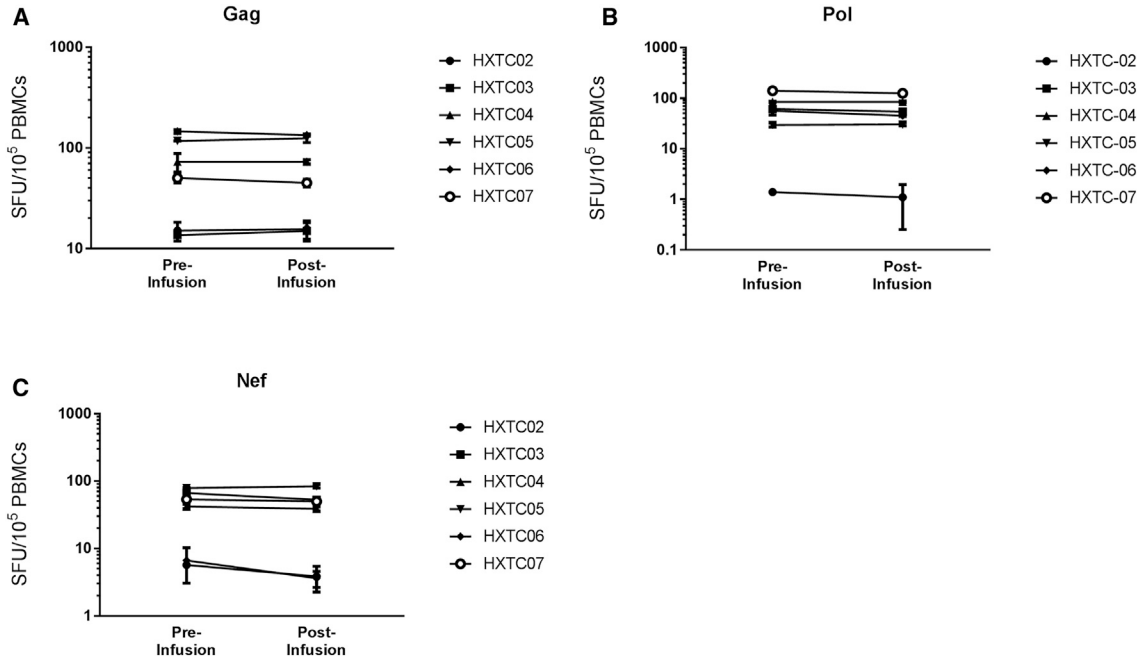

Figure 4. HIV-Specific T Cell Responses following HXTC Infusion by IFNγ Release

PBMCs isolated from leukapheresis products were stimulated with overlapping peptides spanning HIV Clade B Gag (A), Pol (B), or Nef (C) overnight in an ELISpot assay. Resulting IFNγ spot-forming units per 1×10^5 PBMCs are displayed. Error bars represent SEM of n = 4 replicates.

Because the majority of the HXTCs were composed of CD8 T cells, CD8 T cells were the focus of analysis in this manuscript. However, additional cell subsets were present in the HXTC product to varying degrees, including NK cells, in particular in two of the participants. This may be a reflection of the use of modified K562 cells during the expansion process. Recent studies have shown that particular NK cell phenotypes and functional signatures have been associated with improved viremic control and lower reservoir sizes,[60,61] and that NK cells are capable of migrating into B cell follicles during simian immunodeficiency virus (SIV) infection,[62] sparking interest in how to best enhance and employ NK cells for HIV cure.[11,62–64] Further investigation into characterizing the NK cells within the product and post-infusion in future studies may bear interesting results to help inform these efforts.

We did not observe any overall enhancement of the magnitude of the HIV-specific immune response, by IFNγ release or by VIA, when evaluating participants in aggregate. This is not unexpected given the low dose (2×10^7 cells/m^2 per infusion) of the two infusions and the absence of *in vivo* stimulus for expansion of the cells, including the lack of concomitant robust HIV antigen exposure, no pre-infusion lymphodepletion with myeloablative therapy and conditioning, and no additional cytokine support that would promote *in vivo* expansion of the cells. Notably, in the EBV setting, increasing the dose beyond the total dose of 4×10^7 cells/m^2 as used in this HIV study did not translate to an increase in efficacy. This suggests that simply increasing the cell dose may not improve immunologic or clinical impact, and additional strategies to promote *in vivo* expansion may be necessary.

The lack of genetic engineering of T cells allows for an attractive safety profile. However, a limitation of this approach is the inability to precisely track the migration, proliferation, and survival of HXTCs *in vivo*. *Ex vivo*-expanded EBV-specific cells expanded under similar conditions were found to survive for over 4 years following infusion in patients with lymphoma, suggesting that HXTC therapy could have similar longevity.[38] However, extrapolation of these findings from the setting of EBV-mediated lymphoma or hematopoietic stem cell transplant to that of HIV infection is uncertain.

Homing receptor expression on the HXTCs suggests the potential for homing to the gut, due to the high expression of the integrin α4β7 that is increased beyond what was seen in memory CD8 T cells of the corresponding PBMCs. Integrin α4β7 expression is known to be increased by up to 2-fold following exposure to supraphysiologic levels of interleukin-7 (IL-7; albeit largely in the naive subpopulation of CD4 and CD8 T cells), as was used in this particular expansion protocol, which may explain the high levels of α4β7 expression.[65] In contrast, the lymph node homing marker L-selectin (CD62L), which facilitates entry into the lymph node via the high endothelial venules,[66] and the B cell follicle homing chemokine receptor CXCR5, which allows T cells to migrate to B cell follicles within the lymph node along a CXCL13 axis,[67] showed a trend to modest downregulation on HXTCs when compared with memory CD8 T cells in PBMC

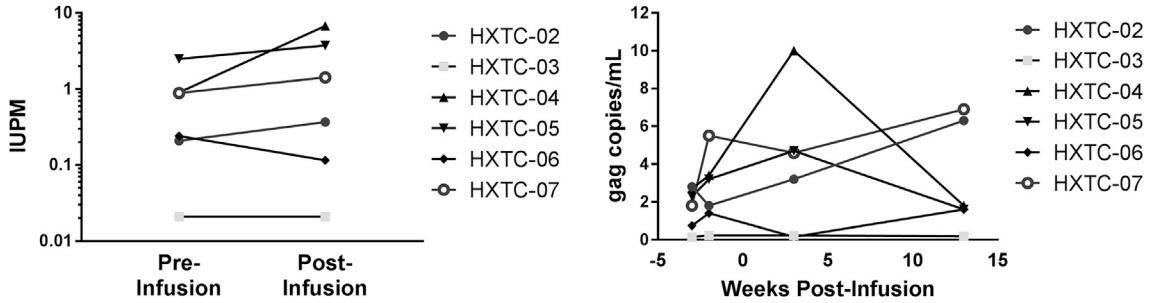

Figure 5. Neither the Latent HIV Reservoir Nor Residual Viremia Decreased following HXTC Infusion

(Left) QVOA was performed to determine the frequency of latently infected resting CD4 T cells using fresh resting CD4 T cells obtained from the baseline week 0 (pre-infusion) and week 13 (post-infusion). Results are presented as infectious units per million (IUPM) CD4 T cells. (Right) The number of copies of HIV-1 Gag RNA/mL of plasma is shown at the indicated time points. Gray shaded symbols indicate that results were below the limit of detection of the assay (values imputed were the threshold values).

counterparts. This is consistent with what was observed in a preclinical evaluation of HXTCs.[41] Although IL-15 has been shown to induce CXCR5 expression[68] and was included during the HXTC expansion, it is possible the overall activating signals may have driven down expression of CXCR5 and/or the IL-15 impact was only transient. Future work could include approaches to durably increase CXCR5 expression on the HXTCs, including optimization of the IL-15 exposure during the expansion process, among other strategies,[69,70] to improve targeting the B cell follicles, a putative site of persistent HIV infection.[71] It is worth noting, however, that although downregulated, the expressions of CD62L and CXCR5 were present; what proportion of the HXTCs homing to lymph nodes is ideal for cure strategies is not yet clear.

As expected with this study design, we did not find any decrease in the size of the latent reservoir, as measured by QVOA. Additionally, we did not observe any significant change in low-level residual viremia by SCA at weeks 3 and 13 following the first infusion. There is the possibility that a modest decline in this measure of persistent HIV infection was unmeasurable given the known visit-to-visit fluctuation in SCA values.[4,59] However, it is most likely that the lack of an impact on reservoir size was due to the absence of therapies designed to perturb the latent reservoir and induce recognizable expression of HIV antigen.

A critical question will be whether HXTC therapy in combination with latency reversing agents can deplete the HIV reservoir to an extent that is measurable by current gold standard assays of HIV latency. A 6-fold reduction measured by the current QVOA assay would hold relevance for HIV cure strategies.[4] Preclinical *in vitro* studies suggest HXTC with the latency reversing agent vorinostat is a promising approach.[42] A study of HXTC in combination with vorinostat is currently undergoing evaluation in an ongoing clinical trial (NCT03212989). Should antigen production following LRA exposure prove to be insufficient to drive *in vivo* expansion of HXTCs, future studies may also need to incorporate strategies such as optimized lymphodepletion therapies or adjunctive cytokine therapy.

MATERIALS AND METHODS

Clinical Protocol

In this phase I single-site study, participants between the ages of 18 and 65 years with a documented history of HIV infection on suppressive ART for at least 1 year were eligible. Participants initiating therapy during either chronic HIV infection (CHI) or acute HIV infection (AHI) were eligible. AHI was defined as: (1) a negative or indeterminate enzyme immunoassay (EIA) plus a reproducibly detectable HIV by amplification methods, or (2) a positive fourth-generation HIV antigen/antibody (Ag/Ab) combination assay and either a negative/indeterminate HIV rapid test or a negative/indeterminate western blot, or (3) a negative HIV RNA test within 45 days of a positive EIA and ART initiation.

Notably, the HIV definitions above are pertinent to the time of diagnosis and treatment initiation. Active co-infections, such as HBV and HCV, and prior receipt of an HIV vaccine and/or immunotherapy within the past 12 months excluded potential participants. Once enrolled, all participants continued baseline ART, completed a baseline leukapheresis procedure, and received two $2 \times 10^7/m^2$ doses of HXTC infusions 2 weeks apart. The study collected serial blood samples throughout to measure immune response by IFNγ ELISpot and VIA, and to measure HIV RNA by SCA. Post-infusion assessments, based on the date of the first infusion, included a leukapheresis performed at week 13 and safety evaluation visits through week 48.

The study was approved by the University of North Carolina, Baylor College of Medicine, and Children's National Medical Center Institutional Biomedical Review Boards, and written informed consent was obtained from each participant.

Generation of HXTCs

HXTCs were generated as previously described.[42,72] In brief, monocytes were isolated from PBMCs by plastic adherence. Dendritic cells were generated using standard cytokine cocktails of IL-4, IL-1β, tumor necrosis factor alpha (TNF-α; R&D Systems, Minneapolis, MN, USA), IL-6 (Cellgenix, Freiburg, Germany), granulocyte-macrophage colony-stimulating factor (GM-CSF) (Sanofi,

Bridgewater, NJ, USA), and prostaglandin E1 (PGE-1) (Pfizer, New York, NY, USA). Non-adherent PBMCs were cryopreserved and thawed upon completion of dendritic cell maturation. PBMCs were stimulated with irradiated autologous dendritic cells pulsed with Gag, Pol, and Nef peptide pools in the presence of IL-7, IL-12 (R&D Systems), and IL-15 (Cellgenix) for the first stimulation. Cells were then co-cultured with autologous PHA-treated blasts pulsed with Gag, Pol, and Nef peptide pools in the presence of IL-15 (Cellgenix) for second stimulation and IL-2 (Prometheus, San Diego, CA, USA) for the optional third stimulation. HIV peptide pools comprised overlapping 15-mer peptides by 11 amino acids, providing coverage of Gag p24, Pol, and Nef across HIV-1 clades (JPT Peptide Technologies, Acton, MA, USA). Irradiated K562 cells (kind gift of Cell Medica, UK) modified to overexpress the co-stimulatory molecules CD80, CD83, CD86, and 4-1BBL were used in the second stimulation and in the optional third stimulation. Expansions were performed in the presence of indinavir and raltegravir (Merck, Kenilworth, NJ, USA) to prevent replication of HIV.

IFNγ ELISpot Assays

Participant PBMCs, cryopreserved at the designated time points pre- and post-infusion, or HXTCs as indicated, were thawed and rested overnight for 16–24 hr in RPMI 1640 with 10% fetal bovine serum (FBS) and 1% glutamine. Cells were then harvested, recounted, and plated at 4×10^5 cells/well in quadruplicate (PBMCs) or 1×10^5 in triplicate (HXTCs) and stimulated with either Gag, Pol, or Nef peptide pools (NIH AIDS Reagent Repository, Bethesda, MD, USA) at a final concentration of 2 μg/mL for 18–20 hr. For each assay, at least four negative control wells (no peptide, 'Mock,' or actin, with HXTC evaluations) and two positive control wells (PHA at a final concentration of 10 μg/mL, for PBMC tests, and staphylococcal enterotoxin B (SEB) for HXTC evaluation; Sigma-Aldrich, St. Louis, MO, USA) were included. For PBMCs, a threshold of 4× Mock and greater than 5 SFU/10^5 was used to determine positive responses, as previously reported by others.[14,43,73] For HXTCs, a threshold of 4× negative control, actin SFU, and greater than 50 SFU/10^5 was used to determine positive responses.

Flow Cytometric Analysis

HXTC clinical products were phenotyped with antibodies (Miltenyi Biotech, Germany) staining for CD3 PerCP Vio700 (BW264/56), CD4 phycoerythrin (PE) Vio770 (M-T321), CD8 allophycocyanin (APC) Vio700 (BW135/80), CD45 VioGreen (5B1), CD56 PE (REA196), CD16 PE (REA423), CD14 VioBlue (TUK4), CD83 APC (HB15), human leukocyte antigen (HLA)-DR fluorescein isothiocyanate (FITC) (AC122), CD32 FITC (2E1), PD-1-PE-Vio770 (PD1.3.1.3), LAG-3-APC (REA351), and TIM-3-PE (F38-2E2), and run on a MACSQUANT Flow Cytometer (Miltenyi Biotech, Germany). Homing receptor markers were performed with staining for CD3 BUV395 (SK7), CD4 BUV496 (SK3), and CD8 BUV805 (SK1) from BD Biosciences (San Jose, CA, USA); CXCR5 APC (MU5UBEE) from eBioscience (Waltham, MA, USA); and CD45RO af488 (UCHL1), CCR7 BV605 (G043H7), CD62L BV421 (DREG-56), and Integrin-β7 PE (FIB27) from BioLegend (San Diego, CA, USA). A live/dead exclusion marker (Zombie Aqua; BioLegend) and staining for exclusion markers (CD16, 3G8, CD56, HCD56, CD14, M5E2, γδ, B1, CD19, HIB19-BV510; BioLegend) were also employed. Samples were analyzed on a BD LSRFortessa Flow Cytometer (BD Biosciences).

Samples were run in parallel with appropriately labeled isotype-matched antibodies and FMOs for gating strategies. Data were analyzed with FlowJo Software (FlowJo, Ashland, OR, USA).

^{51}Chromium Cytotoxicity Assay

The cytolytic activity of HXTCs was assessed in ^{51}Cr sodium chromate (CCS) (^{51}Cr)-release assays 10 days after the final stimulation. Untreated autologous PHA blasts (negative control) or PHA blasts pulsed with Gag, Nef, and Pol pepmixes (JPT) were labeled with CCS for 1 hr at 37°C, washed, and resuspended with HXTCs at E:T ratios from 40:1 to 5:1 and incubated for 4–8 hr. Spontaneous lysis was determined by measuring ^{51}Cr release from target cells into the supernatant using a MicroBeta2 gamma counter (Perkin Elmer, Waltham, MA, USA) in the absence of effectors. Maximum lysis was measured by addition of 1% Triton X-100 (Sigma-Aldrich) to target cells during the 4- to 8-hr incubation. Specific lysis was calculated as follows: Specific lysis % = (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100.

VIA

PBMCs were isolated from participants by peripheral blood draws at the indicated time points by Ficoll isolation. PBMCs were frozen and batched to allow all time points from each participant to be run simultaneously. CD8 T cells were isolated from PBMCs by positive selection (EasySep human CD8 Selection Kit; STEMCELL, Cambridge, MA, USA). CD8-depleted PBMCs were preactivated with 2 μg/mL PHA (Remel, Lenexa, KS, USA) and 60 U/mL IL-2, and spinoculated at 2,500 rpm for 90 min with JR-CSF at an MOI of 0.005. A total of 5×10^4 targets/well was plated in quadruplicate and co-cultured with HXTCs (E:T ratio of 1:10), autologous unexpanded CD8 T cells (E:T ratio of 1:10), or no effectors, in 0.2 mL of RPMI 1640 media supplemented with 10% FBS, 1% penicillin/streptomycin, and 5 U/mL IL-2. Supernatant was assayed on day 6 by p24 ELISA (ABL, Rockville, MD, USA). Results were calculated as %p24 production, normalized to the p24 supernatant concentration in the condition with no effector cells [(concentration p24 in effector wells)/(concentration p24 in control well)*100].

LCA

The LCA was performed as previously described.[42,54,55] In brief, resting CD4 T cells were isolated from a leukapheresis product by negative selection and exposed to vorinostat (VOR) (335n; gift of Merck Research Laboratories, Kenilworth, NJ, USA) at a concentration of 5 million/mL in the presence of the antiretrovirals abacavir (4 μM; gift of Glaxo SmithKline, Philadelphia, PA, USA) and raltegravir (1 μM; gift of Merck Research Laboratories, Kenilworth, NJ, USA). After 6 hr, cells were extensively washed, and CD8 T cells were added

at an E:T ratio of 1:10. Antiretrovirals were again added, and cells were co-cultured at a concentration of 5 million resting CD4 T cells/mL. After 24 hr, cells were extensively washed and depleted of CD8 T cells via three serial magnetic selections (STEMCELL Technologies, Vancouver, BC, Canada). Remaining CD4 T cells were recounted, then plated in 16–48 replicate wells at 0.25–1 million cells/well (depending on cell availability and reservoir size) and cultured with allogeneic CD8-depleted PBMCs from an HIV-negative donor to amplify any residual virus. The presence of HIV *Gag* p24 antigen was measured on day 15 and confirmed on day 19 for each well by ELISA (ABL, Rockville, MD, USA). Each LCA was performed at an independent time point.

Quantitative Viral Outgrowth Assay

Lymphocytes were obtained by continuous-flow leukapheresis. Isolation of resting CD4 T cells and recovery and quantification of replication-competent virus were performed as described previously.[45] In brief, approximately 34–49 million resting CD4 T cells were plated in replicate-limiting dilutions of 2.5 million (12–18 cultures in 6-well plates), 0.5 million (6 cultures in 12-well plates), and 0.1 million (6 cultures in 12-well plates) cells per well, activated with PHA (Remel, Lenexa, KS, USA) and a 5-fold excess of allogeneic irradiated PBMCs from a seronegative donor and 60 U/mL IL-2 for 24 hr. Cultures were washed and co-cultivated with CD8-depleted PBMCs collected from selected HIV-seronegative donors screened for adequate CCR5 expression. Culture supernatants were harvested on days 15 and 19, and assayed for virus production by HIV p24 antigen capture ELISA (ABL, Rockville, MD, USA). Cultures were scored as positive if p24 was detected at day 15 and was increased in concentration at day 19. The number of resting CD4 T cells in IUPM was estimated by a maximum likelihood method.[74,75]

Statistical Analysis

Statistical comparisons between groups were analyzed using the Wilcoxon signed rank test or Friedman's test with posttest correction for multiple comparisons when results were taken in aggregate across participants. Significance for each individual participant was analyzed using a Dunnett's test with posttest correction for multiple comparisons (VIA) or Fisher's exact test (LCA). $p < 0.05$ was considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and one table and can be found with this article online at https://doi.org/10.1016/j.ymthe.2018.08.015.

AUTHOR CONTRIBUTIONS

J.A.S. and S.P. designed, performed, and analyzed results of experiments with assistance from M.L.C. and input from D.M.M., N.G., N.A., and C.M.B. S.P., L.R., T.T., P.J.H., C.R.C., C.M.R., and C.M.B. were responsible for designing and performing expansions of cells. J.A.S., J.D.K., C.L.G., J.J.E., and D.M.M. were responsible for writing, overseeing, and implementing the study protocol. All authors contributed to the writing and editing of the manuscript.

CONFLICTS OF INTEREST

The authors have no conflicts of interest.

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